Journal of Applied Horticulture, 9(1): 3-8, January-June, 2007

Ectopic expression of Mn-SOD in *Lycopersicon esculentum* leads to enhanced tolerance to salt and oxidative stress

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Abstract

Production of reactive oxygen species (ROS) is associated with a number of physiological disorders in plants. Superoxide dismutase (SOD) catalyzes the breakdown of superoxide (O_2^-) into O_2 and H_2O_2 and provides the first line of defense against the toxic effects of elevated levels of ROS. The effect of increased expression of Mn superoxide dismutase (Mn-SOD) on salt stress tolerance was studied using transformed tomato (*Lycopersicon esculentum* cv. Zhongshu No. 5) plants. Northern blots confirmed expression of the heterologous Mn-SOD in transgenic plants. Strong Mn-SOD enzyme activity was detected by native PAGE in transformed plants. Transgenic plants showed resistance to the superoxide-generating herbicide methyl viologen (MV, 10^{-4} M). The total SOD activity was one and one half- to two-fold higher, and APX (ascorbate peroxidase) activity was six to seven fold higher in transgenic, than in wild-type (WT) plant under MV stress. Germination of transgenic tomato seeds at a NaCl concentration of 150 mM was greater than wild-type seeds. When exposed to salt stress, roots of transgenic plants were less stunted and leaf injury was lower than that observed in WT plants. Also, the total APX activity of transgenic plants was 4 to 5 fold higher than that of WT under NaCl (200 mM) stress.

Key words: Superoxide dismutase, oxidative stress, salt stress, transgenic tomato.

Introduction

Salt stress is one of the most common types of abiotic stress that limits the production of agricultural plants around the world (Zhu, 2000). Plants subjected to salt stress also show elevated levels of activated forms of oxygen and free radicals. These elements often are associated with damage to membranes and essential macro-molecules such as protein, DNA, and lipids (Fadzilla *et al.*, 1997; Gueta-Dahan *et al.*, 1997; Hernández *et al.*, 1995).

Superoxide dismutase (SOD) is an important enzyme in a plant's defense against oxidative stress. It catalyzes the conversion of two superoxide anions (O_2^-) into hydrogen peroxide (H_2O_2) and O_2 and alleviates oxidative stress (Bowler *et al.*, 1992). SODs are a group of metal-containing enzymes and are classified into three types according to their metal cofactor requirements: iron SOD (Fe-SOD) is localized in the chloroplast; copper-zinc SOD (Cu/Zn-SOD) is localized in the chloroplast, cytosol, and possibly the extracellular space; and manganese SOD (Mn-SOD) is found mainly in mitochondria and peroxisomes (Alscher *et al.*, 2002).

Antioxidant enzyme activity is found in plants responding to various environmental and chemical stresses (Allen, 1995; Baek *et al.*, 2006), such as freezing (Martinez *et al.*, 2001), chilling (Baek and Skinner, 2005; Iannelli *et al.*, 1999), salt (Gueta-Dahan *et al.*, 1997; Hernández *et al.*, 1995; Rajguru *et al.*, 1999), and methyl viologen (MV) (Bowler *et al.*, 1991; Donahue *et al.*, 1997).

The role of SOD during salt stress has received much attention. Exposure of salt-tolerant pea plants to NaCl resulted in the formation of O_2^- and H_2O_2 and increased the activity of SOD and other antioxidant enzymes, such as ascorbate peroxides (APX). Transcripts levels for Mn-SOD, Cu/Zn-SOD, and APX were strongly induced in the salt-tolerant variety but not in the salt-sensitive one (Hernández *et al.*, 2000). Reports dealing with rice (Dionisio-Sese and Tobita, 1998) and tomato plant (Shalata *et al.*, 2001), have also reported increased SOD activity in salt-tolerant cultivars when exposed to salt stress. Additionally, Tanaka *et al.* (1999) confirmed that overexpression of a yeast *Mn-SOD* gene in rice confers tolerance to salt stress.

Enhanced expression of SODs in transgenic plants has demonstrated tolerance to MV (Allen, 1995; Perl *et al.*, 1993), freezing (McKersie *et al.*, 1999), and salt (Tanaka *et al.*, 1999). In order to further understand the role of APX and SOD in response to oxidative stress induced by abiotic stresses, transgenic tomato plants were produced that overexpress either cAPX (Wang *et al.*, 2005 and 2006) or Mn-SOD and their response to several abiotic stresses was evaluated. The purpose of this study was to evaluate the tolerance of transgenic *Mn-SOD* tomato plants to salt (NaCl) and oxidative (MV) stress.

Materials and methods

Generation and analysis of transgenic tomato plants: Mn-SOD cDNA was synthesized from rubber tissue (*Hevea brasiliensis*) based on primers by Miao and Gaynor (1993). The cDNA was mobilized into the binary vector pDU92.3103 (Tao *et al.*, 1995) between the cauliflower mosaic virus 35S promoter and

terminator regions. The construct, pDU96.2144, contained the β-glucuronidase (GUS) reporter gene and the *NPTII* selectable marker, and was transferred into the tomato (*Lycopersicon esculentum* cv. Zhongshu No. 5) genome via the *Agrobacterium tumefaciens*-mediated transformation method described by Frary and Earle (1996) with modifications by Wang (2005). The kanamycin-resistant transformants were screened by the GUS histochemical staining assay according to the method developed by Jefferson *et al.* (1987). Genomic DNA was isolated from wild-type (WT) and *Mn-SOD*-overexpressing plants (T_1), according to the procedure of Doyle and Doyle (1987).

PCR reaction used the 35S promoter forward primer and rubber *Mn-SOD*-specific reverse primer (Miao *et al.*, 1993). The sequences for the forward and reverse primers for 35S::*Mn-SOD* included 5'– CACGTCTTCAAAGCAAGTGG –3' and 5'– CTAAGAAGAAGGGCATTCTTTGGCAT –3', respectively. About 20 ng DNA was used for the PCR reaction, under the following conditions: 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C for 30 cycles.

Mn-SOD expression was assayed by northern analysis and SOD activity gel assay. Wild-type (WT) and transgenic plants were grown in a greenhouse under natural lighting supplemented with sodium vapor lamps (1000W, Philips, Inc., Eindhoven, Netherlands) for a 16-h photoperiod at approximately 23/21ºC (± 2ºC, day/night temperature). Seedlings were grown in a potting mix containing peat moss (Premier Horticulture, Inc., Quebec, Canada). T_1 seeds obtained by self-pollination of each of T_0 plants were screened for resistance to kanamycin. T_2 seeds were obtained from individual T_1 plants by self-pollination and were used to generate T_2 transgenic progeny. All the transgenic plants were resistant to the kanamycin.

Northern Blot assay: Total RNA was extracted from leaves of transgenic and WT plants using Tri-reagent (Molecular Research, Inc.). 30 µg of total RNA from each sample was used for northern blot assay. The hybridization procedure was as described by Super Hyb kit (Molecular Research, Inc., Cincinnati, OH). The hybridization probe was a ^{32}P-labeled Mn-SOD PCR fragment. RNA hybridization was detected using a PhosphorImager SI (Molecular Dynamics Inc., Sunnyvale, CA).

APX and SOD gel activity assay: About 100 mg of leaf tissue was ground to a fine powder in liquid nitrogen and homogenized in 200 µl of APX activity gel grinding buffer [100 mM $NaPO_4$ (pH 7.0), 5 mM ascorbate, 1 mM EDTA (pH 8.0), 10% glycerol, and 0.001% bromophenol blue] and SOD activity gel grinding buffer [50 mM Tris (pH 6.8), 10% glycerol, and 0.001% bromophenol blue], respectively. The supernatant was collected and protein concentration was determined using a Protein Assay system (Bio-Rad, Hercules, CA). 70 µg of total protein was loaded into each lane of a native PAGE gel. APX gel activity analysis was conducted as described by Mittler and Zilinska (1993). The SOD gel activity assay was described by Payton *et al.* (1997). After staining, the gels were scanned with a Densitometer Scanner I (Molecular Dynamics, Inc., Cincinnati, OH). The bands were analyzed using ImageQuant 5.2 software (Molecular Dynamics, Inc., Cincinnati, OH).

Application of methyl viologen: Shoots were treated with methyl viologen (MV, Sigma) following the procedure described by Perl *et al.* (1993). Shoot cuttings (with three to four leaves) from WT and T_0 transgenic plants were obtained from containerized mature plants grown in the greenhouse. The cut end of the shoots was placed in 10^{-4} M MV. After 16 h, the cut ends were transferred to tap water for an additional two days. Two leaf discs (1.02 cm in diameter) of the third leaf from the apex were excised after the MV treatment, and MV-induced oxidative damage was evaluated using the leaf electrolyte leakage (Wisniewski *et al.*, 1997) with a conductivity meter (Markson Science, Inc., Del Mar, CA). Means for all values were an average of two subsamples in each plant with three replications. The data were subjected to Duncan's multiple range test (NCSS-PASS software, NCSS Inc., Williamsport, PA). Arcsine square root transformations were performed before data analysis; nontransformed means are presented. MV treated leaf discs were also sampled and stored at –80°C prior to APX and SOD analyses.

Salt Stress: To evaluate salt stress, seeds from WT and T_1 plants were placed in petri dishes (≈40 seeds per box) on filter paper (Whatman 3MM) saturated with 150 mM NaCl solution. They were germinated at 23/21°C (± 2°C, day/night temperature) under cool white fluorescent lights (100-150 µmol m^{-2} sec^{-1}) using a 16-h photoperiod. At the end of two weeks, germination was measured. Germination was considered successful when the radicle protruded through the seed coat. Values are means ± SE (three repeats). The data were subjected to Duncan's multiple range test.

To evaluate the tolerance of developing roots to salt stress, shoot cuttings from 10-day-old WT and T_2 seedlings were grown in sterile, solidified Murashige-Skoog (MS; Murashige and Skoog, 1962) medium (Sigma) amended with either 200 mM or 250 mM NaCl. After 5 weeks at 23/21ºC (± 2ºC, day/night temperature), the fresh roots were excised, blotted on filter paper, and weighed. Values are means ± SE (six replicates). The data were subjected to Duncan's multiple range test.

WT and T_2 shoot cuttings were grown in rooting medium for 2 weeks. Healthy seedlings were transferred to 5.8 × 5.8 × 8-cm plastic pots with peat moss soil and watered with tomato fertilizer (9N-4.4P-12.5K, Schultz, Inc., Bridgeton, MO) for one week. Stress was imposed by watering plants with tomato fertilizer and NaCl (200 mM or 250 mM) solution every three days. Controls received only fertilizer. The plants were maintained in a greenhouse with natural lighting supplemented with sodium vapor lamps (1000W, Philips, Inc., Eindhoven, Netherlands) for 16-h photoperiod at approximately 23/21°C (± 2°C, day/night temperature). Height (from the apex to soil) was measured after 10 days (d). The extent of injury was evaluated visually at 10, 20, and 30 d following treatment. The scale was as follows: 0, no injury; 1, slow growth but no obvious damage, ≤20% leaf area exhibited injury; 2, leaves turned yellow, 21 to 40% of the leaf area injured; 3, plants wilted, 41 to 60% leaf area injured; 4, seriously damaged, the plant became soft and could not remain upright, 61 to 80% leaf area injured; 5, 81 to100% leaf area injured or plant died. Mean values of 5 replicates were calculated and the ranks were subjected to the non-parametric Kruskal-Wallis test. The leaf discs from WT and transgenic plants grown for 10-d under NaCl (200 mM) stress were then frozen in liquid nitrogen and stored at –80 ºC for further enzyme activity gel analyses.

Results

Overexpression of the Mn-SOD gene: No detectable GUS activity was seen in WT leaves. Transgenic leaves stained intensely blue, indicating high levels of GUS activity. No PCR products could be amplified from WT plants, whereas products of the expected size (≈800 bp) were amplified from all transgenic lines (date not shown). Northern-blot analysis was performed to assess the mRNA levels in transgenic plants. All the transgenic plants contained transcripts but levels among individual lines varied. WT plants did not exhibit transcript that hybridized to the probe (Fig. 1). Two transgenic lines, S4 and S20, were selected for further study because they showed higher expression levels.

The selected transgenic lines were screened by gel assay for the presence of Mn-SOD activity. Two isoforms were observed in all plants, and may represent chloroplastic (chl) and cytosolic (cyt) Cu/Zn-SOD (Perl *et al.*, 1993). Transgenic plants displayed an additional lower mobility band corresponding to the Mn-SOD enzyme activity (Fig. 2A). Total APX activity was also measured.

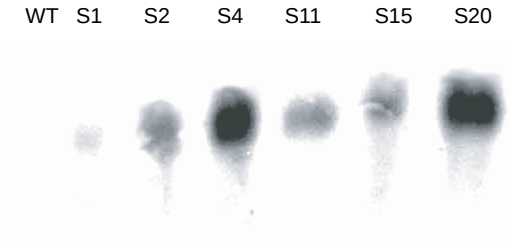

Fig.1. Northern blot analysis of RNA isolated from the T_1 plants. 30 µg of total RNA was used per lane for each blot. Blots were probed with ^{32}P-labeled Mn-SOD PCR products. WT, wild-type plant; S1 to S20, independent transgenic lines.

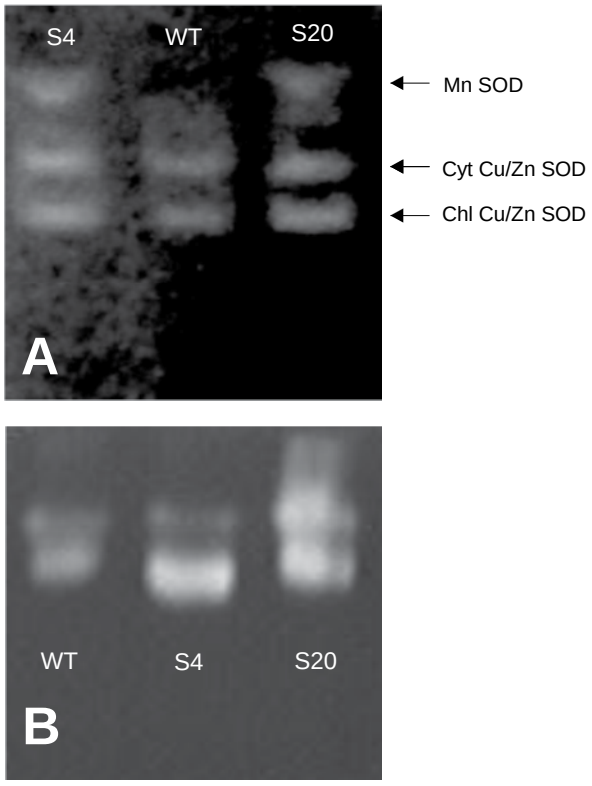

Fig. 2. Detection of (A) SOD and (B) APX activity in leaves of wild-type (WT) and transgenic Mn-SOD (Lines S4, S20) tomato plants. About 70 µg protein was loaded on the non-denaturing polyacrylamide gel.

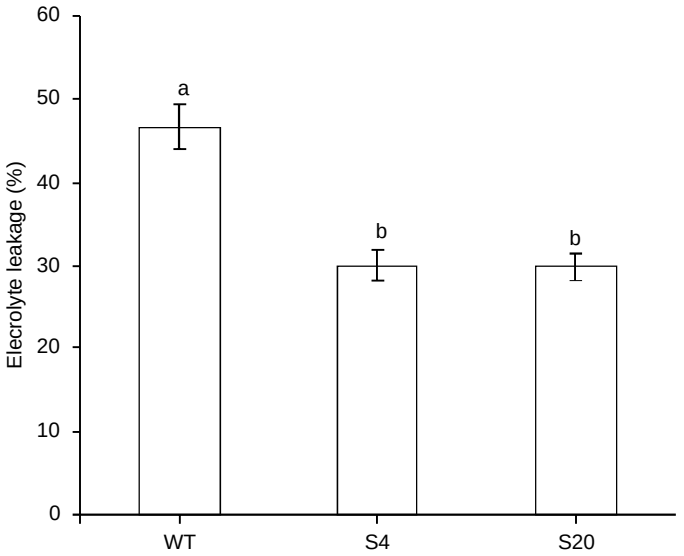

Fig. 3. Effects of methyl viologen (10^{-4} M) stress on electrolyte leakage from transgenic Mn-SOD (Lines S4, S20) and wild-type (WT) tomato plants. Bars represent SE for three replicates. Different letters indicate significant differences (P<0.05) between means (Duncan's multiple range test).

Lines S4 and S20 exhibited 2- to 3-fold higher APX activity than in WT plants (Fig. 2B).

Effects of MV stress: Methyl viologen-induced electrolyte leakage in transgenic plants was significantly less than in WT (P<0.05, Fig. 3). Mean electrolyte leakage in WT leaves was about 47%. In comparison, Mn-SOD-expressing lines (S4, S20) had significantly lower electrolyte leakage (approximately, 30%). After two days under MV treatment, total SOD activity was 1.5- to 2-fold higher in transgenic plants. APX activity of transgenic plants was 6- to 7-fold higher than WT plants following MV treatment (data not shown).

Effects of NaCl stress: The effect of 150 mM NaCl on the percent seed germination at 13 days was 76 and 81% germination for transgenic line S20 and S4, respectively and 39% for WT seeds (Fig. 4). After 5 weeks of treatment, the fresh root weight of transgenic plant (14 g and 8 g for S4; 14 g and 7 g for S20) was significantly (P<0.05) greater than that of WT (8 g and 2 g) tomato plants at 200 mM and 250 mM NaCl, respectively (Fig. 5). Irrigation of transgenic and WT tomato plants with 200mM

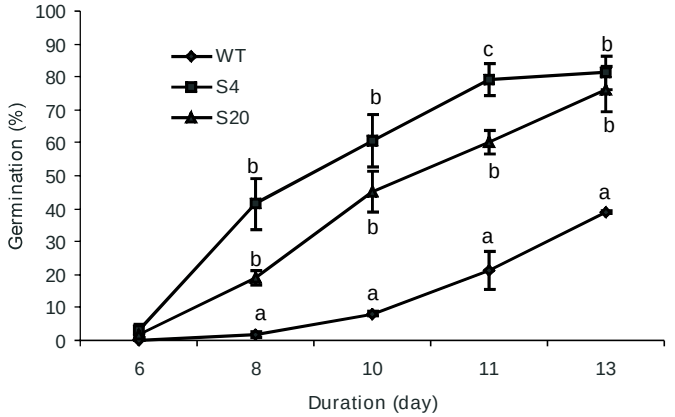

Fig. 4. Germination in T_2 transgenic Mn-SOD (Lines S4, S20) and wild-type tomato seeds treated with NaCl (150 mM) for various periods of time. Values are means ± SE (n=3). Different letters indicate significant differences (P<0.05) between means within each sampling time (Duncan's multiple range test).

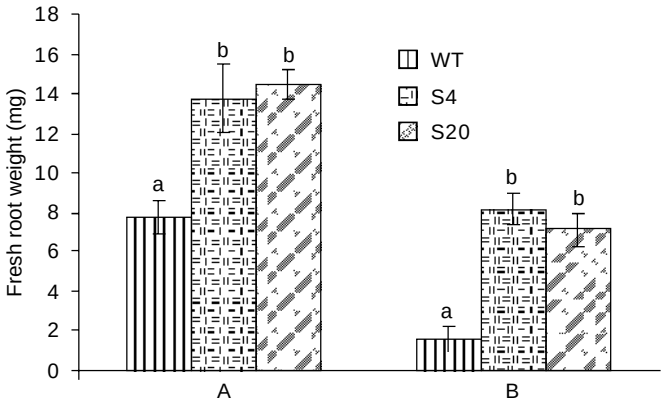

Fig. 5. Effects of 200 mM (A) or 250 mM (B) NaCl treatments on root development of wild-type (WT) and transgenic Mn-SOD (Lines S4, S20) tomato plants. Fresh weight was determined 5 weeks after the treatment. Values are means ± SE (n=6). Different letters indicate significant differences (P<0.05) between means within each NaCl treatment level (Duncan's multiple range test).

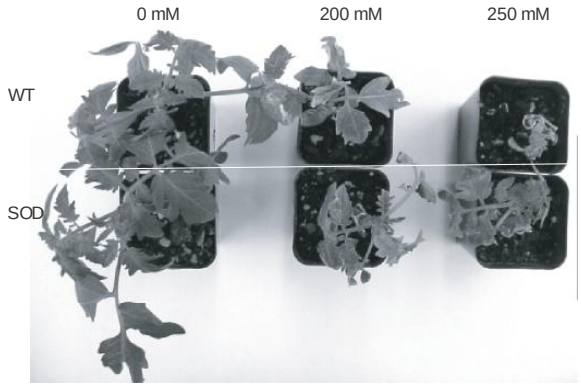

Fig. 6. Effects of salt stress on wild-type (WT) and transgenic Mn-SOD (Line S20) tomato plants. Plants were treated with 200 mM and 250 mM NaCl for 18 d in the greenhouse.

and 250 mM NaCl solution severely inhibited their shoot height growth (Fig. 6). The margins of leaflets in WT plants became necrotic following 10 days of salt treatment. Visible injury to WT was rated as 2.2. In contrast, injury to transgenic plants was significantly lower (P<0.05), showing only slight leaf area injury (scale 1). The effect of the salt treatment was still apparent but severe after 20 d. WT plants displayed visible necrotic injury (scale 3.6) after 20 d. In contrast, the transgenic seedlings showed less injury (Fig. 7A). At 250 mM NaCl, WT seedlings showed injury scale 5 after 20 d, whereas the transgenic plants exhibited less wilting injury (average scale 2.2 for S4 and 3 for S20). The differences in visible injury between transgenic and WT plants were statistically significant (P<0.05, Fig. 7B). The leaf APX activity in transgenic plants was about 4- to 5-fold higher than that in WT plants after 10 d of NaCl (200 mM) treatment (data not shown).

Discussion

Numerous studies have indicated that oxidative stress enhances SOD activity (Donahue *et al.*, 1997; El-Saht, 1998). Abiotic stresses, such as chilling, drought, and salt stress have been correlated with increase in SOD activity (Baek *et al.*, 2006; Dionisio-Sese and Tobita, 1998; Fadzilla *et al.*, 1997; Scandalios, 1993).

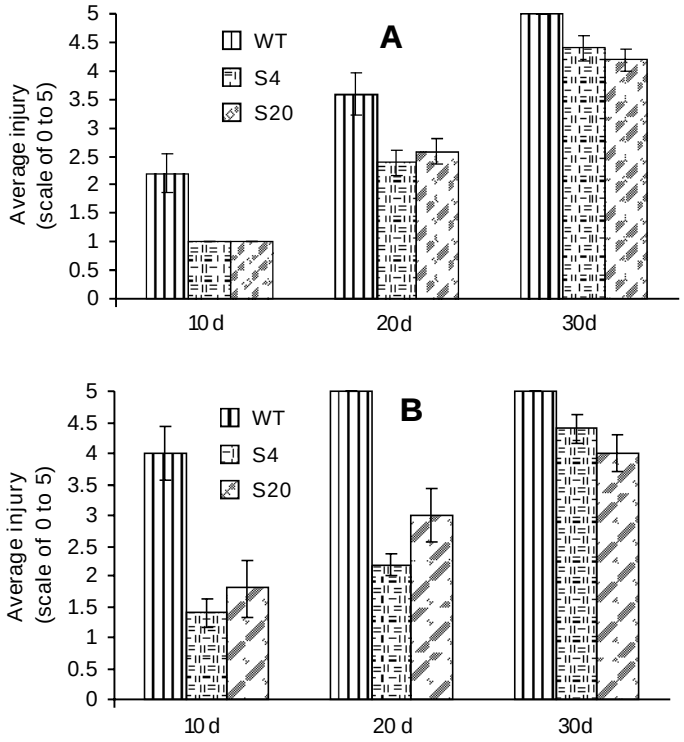

Fig. 7. Injury to wild-type (WT) and transgenic Mn-SOD (Lines S4, S20) tomato plants under stress from (A) 200 mM or (B) 250 mM NaCl treatments after 10, 20, and 30 d. No visible injury = 0; slow growth, and <20% visible injury = 1; yellowing leaves and 21-40% of leaf area with visible injury = 2; wilted plants and 41-60% visible injury = 3; seriously damaged plant unable to remain upright with 61-80% visible injury = 4; dead plant or >80% visible injury = 5. Values are means ± SE (n=5). Different letters indicate significant differences (P< 0.05) between means within each sampling time (Kruskal-Wallis test). *data for WT was also cited in Wang *et al.* (2005).

Our study indicated that transgenic tomato plants expressing the Mn-SOD gene from *Havea* displayed an enhanced tolerance to both MV and salt-induced oxidative stress. Transgenic plants had less electrolyte leakage than WT plants (Fig. 3), suggesting that overexpression of Mn-SOD in the transgenic plants reduced cellular damage caused by ROS (Bowler *et al.*, 1991). After MV treatment, APX and SOD activity was higher in transgenic plants and was related to a plant's resistance to ROS damage (Allen, 1995; Van Camp *et al.*, 1994).

An increase in ROS scavenging capacity is required to enable rapid removal of ROS produced during early seed imbibition (Gidrol *et al.*, 1994). In this study, transgenic seeds were more tolerant to NaCl than WT seeds. The transgenic plants had higher SOD and APX activity, which could prevent the accumulation of O_2^- and H_2O_2 concentration during the early stages of seed germination.

In our study, we found that shoot cuttings of transgenic tomato plants produced more root biomass than WT plants under salinity stress, indicating that overexpression of Mn-SOD enabled the transgenic plants to better deal with oxidative stress. Also, seedlings of transgenic *Mn-SOD* tomato plants showed less injury (Fig. 7) following NaCl (200 mM and 250 mM) stress than WT plants. This is consistent with the theory that increased antioxidant enzyme activity can prevent NaCl-induced oxidative stress (Fadzilla *et al.*, 1997; Tanaka *et al.*, 1999).

Our study showed that APX activity increased due to NaCl treatment and is consistent with other reports (Hernández *et al.*,

1999; Mittova *et al*., 2002; Sairam and Srivastava, 2002; Wang *et al*., 2005, 2006). This confirms earlier reports that APX plays an important role in scavenging H_2O_2 induced by NaCl stress. However, SOD activity decreased after 10 d of NaCl treatment (data not shown). The reason for this decrease in activity is not known but may be related to the long exposure to NaCl.

In this study, the overexpression of Mn-SOD in transgenic tomato plants enhanced seed germination, root development and seedling tolerance to NaCl stress. We conclude that increased antioxidant levels may play an important role in scavenging ROS when plants are exposed to salt stress.

Acknowledgments

We are grateful to the Washington Tree Fruit Research Commission for financial support. We also thank Dr. Lailiang Cheng (Cornell University; Ithaca, NY) for technical advice and assistance.

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